Pure culture synthesis of ectomycorrhizae by *Thelephora terrestris* and *Pisolithus tinctorius* on different conifer hosts

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In a special plant-growth room, isolates of *Thelephora terrestris* produced basidiocarps and formed typical ectomycorrhizae with seedlings of bristlecone, jack, sand, lodgepole, shortleaf, slash, sugar, Austrian, longleaf, cluster, ponderosa, red, pitch, eastern white, Scots, loblolly, and Virginia pines and Douglas fir. Atypical mycorrhizae (lacking mantle) were formed on seedlings of Norway spruce and jack, Japanese red, and Himalayan pines. The formation of atypical mycorrhizae was considered a result of differences in the symbiotic–parasitic nature of the fungal symbionts on different hosts. *Pisolithus tinctorius* formed typical mycorrhizae with seedlings of jack, sand, Japanese red, shortleaf, slash, Austrian, longleaf, cluster, red, pitch, eastern white, Scots, loblolly, and Virginia pines. Reisolation of specific fungal symbionts from mycorrhizae of several pine hosts was successful.

Mycorrhizae formed by *T. terrestris* were macroscopically and microscopically different from those of *P. tinctorius*, but mycorrhizae formed by different isolates of *T. terrestris* were indistinguishable from each other, regardless of host. These results suggest that the fungal symbiont determines color and morphology of ectomycorrhizae.

Introduction

This report expands the host range of *Thelephora terrestris* (Ehrh.) Fr., which has been reported as forming morphologically identical ectomycorrhizae[2] on the roots of 11 pine species (3). We suggested that the fungal symbiont is primarily responsible for the morphology of ectomycorrhizae. *T. terrestris* is a pioneer symbiont colonizer of fumigated (4) and other symbiont-free soils (13) and is found in forests and forest-tree nurseries in many parts of the world (10). *Pisolithus tinctorius* (Pers.) Coker & Couch has been associated with ectomycorrhizae of numerous forest trees including *Eucalyptus* species (12) and is symbiotic with shortleaf and loblolly pines in aseptic culture (1, 5). *P. tinctorius* has an unusually high temperature optimum for growth in pure culture (28 °C) and for mycorrhizal synthesis (34 °C) on aseptic loblolly pine.[3] Basidiocarps of this fungus are frequently found in soils subjected to extremely high temperatures (11).

The ability of *T. terrestris* to colonize fumigated or other symbiont-free soils rapidly and *P. tinctorius* to grow and form mycorrhizae in soils of high temperature renders them potentially valuable as introduced symbionts in reforestation and afforestation. The purpose of this research was to examine the mycorrhizal synthesizing capacity of two isolates of *T. terrestris* and one isolate of *P. tinctorius* on a variety of tree species, and to make detailed macroscopic and microscopic examinations and descriptions of the mycorrhizae formed on these hosts for use in field identification and evaluation of each mycorrhizal association.

Materials and Methods

The fungal symbionts tested were *T. terrestris* isolates 2 and V-9037 and *P. tinctorius* isolate 49. Isolate 49 of *P. tinctorius* was obtained in 1967 from a sporophore collected in a pine plantation near Athens, Georgia. The sources of *T. terrestris* isolates have been previously reported (2, 4). Fungal inocula were 4-month-old cultures grown at 25 °C in 1-liter volumes of vermiculite – peat moss substrate (8) at pH 5.5, moistened with 500 ml of modified Melin-Norkrans (MMN) liquid medium (2).

The conifer hosts used in this experiment are listed in Table I. Seed for shortleaf, loblolly, longleaf, slash, and sand pine hosts were obtained from the Eastern Tree Seed Laboratory, USDA Forest Service, Macon, Georgia; all others were obtained from a commercial tree seed

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[2]Terminology suggested by Peyronel, B., B. Fassi, Anna Fontana, and J. M. Trappe. 1969. Terminology of mycorrhizae. Mycologia. 61: 410–411.

[3]Marx, D. H., W. C. Bryan, and C. B. Davey. 1970. Influence of temperature on aseptic synthesis of ectomycorrhizae by *Thelephora terrestris* and *Pisolithus tinctorius* on loblolly pine. (In preparation for publication, Forest Sci.)

distributor. Seed were soaked in 1% H_2O_2 for 5 days at 5 °C, surface sterilized in 30% H_2O_2 for 20 min, and soaked in sterile water until planted.

The experiment was conducted in a plant-growth room that was electronically air-filtered and air-conditioned. Fumigation, sterility checking, and other operational procedures have been previously described (3). Wooden flats (48 cm × 30 cm × 15 cm) were filled with a homogenous mixture (1:8 v/v) of fungal inoculum and triple-autoclaved soil (loams:and:peat moss: 2 parts vermiculite by volume). Control flats were prepared with fungus-free medium and soil mixture. Treated seed of the hosts were planted in single rows (30 cm) with three flats comprising a replication. Sequence of planting hosts in each flat was determined at random before planting and was the same for each series of replications. Each

TABLE I

Degree of ectomycorrhizal development by *Thelephora terrestris* and *Pisolithus tinctorius* on different conifer hosts

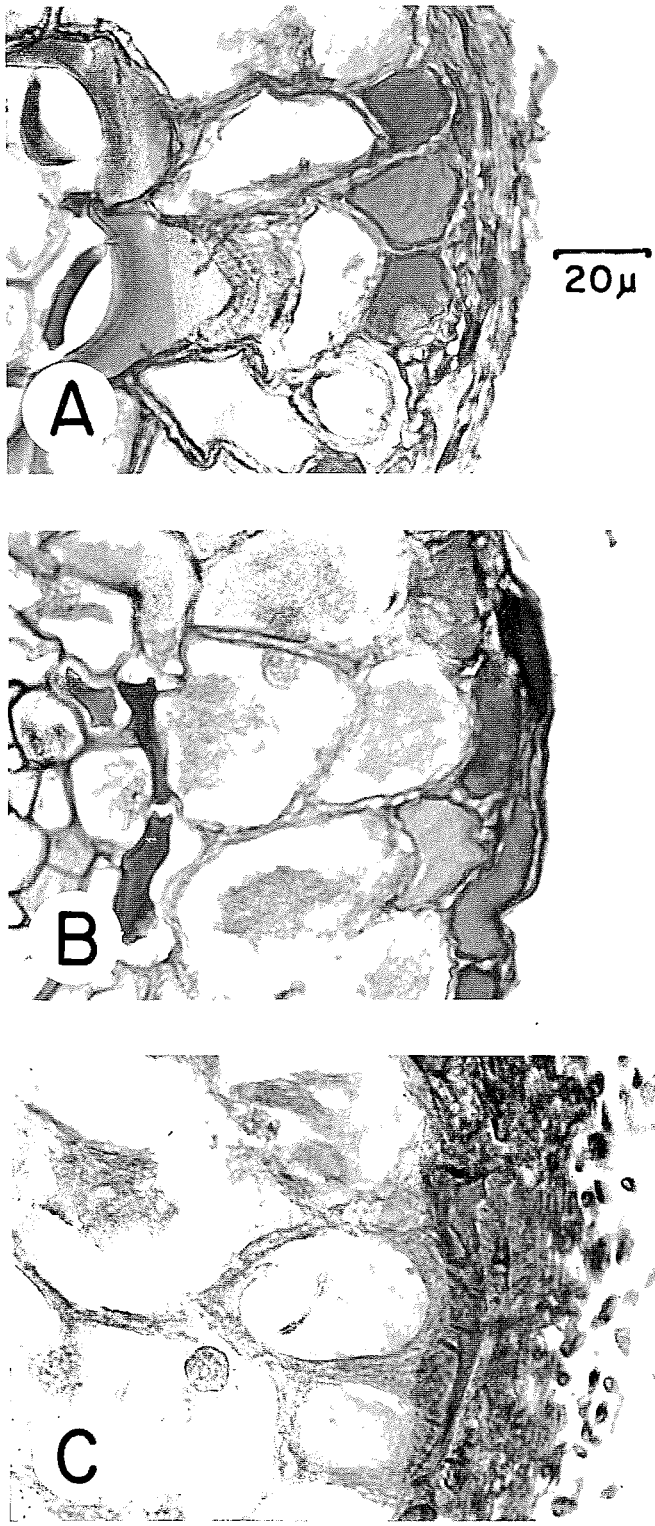
Host	*T. terrestris*, isolate 2	*T. terrestris*, isolate V-9037	*P. tinctorius*, isolate 49
Picea abies (L.) Karst. Norway spruce	None[a]	Poor[b]	None
Pinus aristata Engelm. bristlecone pine	None	Moderate	None
P. banksiana Lam. jack pine	Poor[b]	Poor	Moderate
P. clausa (Chapm.) Vasey sand pine	Poor	Moderate	Poor
P. contorta Dougl. lodgepole pine	Poor	Excellent	None
P. densiflora Sieb. & Zucc. Japanese red pine	Poor[b]	Moderate[b]	Poor
P. echinata Mill. shortleaf pine	Good	Moderate	Poor
P. elliottii Engelm. var. *elliottii* slash pine	Poor	Good	Poor
P. griffithii McClelland Himalayan pine	Poor[b]	None	None
P. lambertiana Dougl. sugar pine	Poor	Poor	None
P. nigra Arnold Austrian pine	Poor	Moderate	Poor
P. palustris Mill. longleaf pine	Moderate	Good	Poor
P. pinaster Ait. cluster pine	Moderate	Moderate	Moderate
P. ponderosa Laws. ponderosa pine	Poor	Moderate	None
P. resinosa Ait. red pine	Moderate	Excellent	Moderate
P. rigida Mill. pitch pine	Poor	Poor	Moderate
P. strobus L. eastern white pine	Moderate	Excellent	Poor
P. sylvestris L. Scots pine	Poor	Good	Moderate
P. taeda L. loblolly pine	Moderate	Poor	Poor
P. virginiana Mill. Virginia pine	Poor	Moderate	Good
Pseudotsuga menziesii (Mirb.) Franco var. *menziesii* Douglas fir	Poor	None	None

[a]Rating based on macroscopic examination of at least 100 seedlings per host. Excellent: about 75 to 100% of feeder roots mycorrhizal; good: 50 to 74%; moderate: 25 to 49%; poor: 1 to 24%, and none: mycorrhizae absent.

[b]Atypical ectomycorrhizae without fungal mantle but with excellent Hartig-net development to the endodermis.

FIG. 1. Ectomycorrhizae of jack pine from pure culture inoculation: (A) typical mycorrhiza formed by *Thelephora terrestris* isolate V-9037, (B) atypical mycorrhiza (without fungal mantle) formed by *T. terrestris* isolate 2, and (C) typical mycorrhiza formed by *Pisolithus tinctorius* isolate 49.

PLATE I



treatment was prepared in duplicate and controls were replicated five times. Two replications of the control were placed on greenhouse benches outside the growth room for comparisons. Flats inside the growth room were completely randomized on benches.

After seeds were planted, soil in each flat was covered with a layer of perlite (1 cm deep) to reflect light. The soil was then saturated with water and thereafter received about 450 ml daily through an automatic watering system. Two months after the seed were planted, 1 liter of Melin-Norkrans (MN) salt solution (2) was added to each flat.

After 18 weeks, the seedlings were lifted from the soil, the roots were gently washed in water, and the mycorrhizal development was visually assessed under 4× magnification. At least 100 seedlings were examined for each host–symbiont or control treatment. About 50 mycorrhizae per treatment were fixed, dehydrated, embedded in hard paraffin, serially sectioned (8 μ), and stained in safranine–fast green (5) for detailed histological examinations. To reisolate the symbionts, additional mycorrhizae were washed in detergent, surface sterilized in 100 p.p.m. $HgCl_2$ for 1½ min, rinsed several times in sterile water, and incubated on MMN agar at 23 °C in diffuse light (4) for several weeks. Isolation was attempted from at least 30 mycorrhizae from each of six different hosts. Roots of control seedlings from the growth room were sliced on glass slides and examined directly at 300× in phloxine and lactophenol.

Soil and air temperatures in the growth room were recorded several times daily with a remote-sensing, thermister-based, telethermometer system (3). During the experiment, soil temperatures averaged 20.9 °C during daytime, with extremes of 14.1 to 28.4 °C for periods of less than 2 h; soil temperatures at night averaged 16.4 °C, with extremes of 16.0 to 17.5 °C. Air temperatures during daylight averaged 27.7 °C, ranging from 21.3 to 34.6 °C; air temperatures at night averaged 17.5 °C, ranging from 15.2 to 18.1 °C. Relative humidity averaged 64% during the day and 52% at night.

Results

In 8 to 10 weeks after seed were planted, basidiocarps of *T. terrestris* developed on seedling stems in flats containing both isolates of this fungus. Basidiocarps were removed immediately to eliminate basidiospore contamination of adjacent seedlings. Basidiocarps of *T. terrestris* also developed on seedling stems in control flats outside the growth room, but did not develop in flats of any other treatment.

Mycorrhizae did not develop on noninoculated control seedlings of any host species in the growth room. Several pine hosts developed dichotomously branched short roots with root hairs, but most had simple short roots with abundant root hairs. Roots of all control seedlings from inside the growth room were extremely succulent and healthy.

All noninoculated control seedlings outside the growth room, except Norway and black spruce, Douglas fir, and bristlecone pine, formed ectomycorrhizae, apparently from airborne inoculum of fungal symbionts. The degree of mycorrhizal development on these control seedlings varied: histological examination revealed at least two different fungal symbionts. One fungus with large hyphae formed ectomycorrhizae on all pine hosts except longleaf, and was identical with the fungal symbiont previously reported (3) in mycorrhizae of six pine species grown under similar conditions. The mycorrhizae were dark brown and primarily bifurcate in structure. Mantle thicknesses varied from 21 to 55 μ (average 37 μ) regardless of host, and the Hartig-net rarely penetrated past the first cortical cell layer. The other symbiont formed mycorrhizae on roots of all pine hosts except Japanese red and lodgepole pines and closely resembled *T. terrestris* in hyphal diameter and clamp connections (4). The mycorrhizae were light brown and primarily coralloid. Mantle thickness varied between 10 and 38 μ (average 19 μ) regardless of host, and the Hartig-net developed to the endodermis.

Thelephora terrestris isolate 2 formed typical ectomycorrhizae with 15 pine species and Douglas fir (Table I). This symbiont also formed mycorrhizae with jack, Japanese red, and Himalayan pines. These mycorrhizae were considered atypical, however, because the fungal mantle was either absent or sparse and without organization. Hartig-net development in atypical mycorrhizae was well defined and penetrated to the endodermis (Fig. 1). *T. terrestris* was reisolated from mycorrhizae of longleaf and eastern white pines and the cultures were physiologically and morphologically identical with the original parent isolate (4). The fungus was not reisolated from mycorrhizae of Japanese red, ponderosa, slash, or shortleaf pines.

Thelephora terrestris isolate V-9037 formed typical ectomycorrhizae with 17 pine species (Fig. 1). In addition, atypical ectomycorrhizae were formed on Norway spruce and Japanese red pine (Table I). The degree of mycorrhizal development by this isolate was generally greater than that of isolate 2 on the same pine species. The fungal symbiont was reisolated from mycorrhizae of jack, shortleaf, and slash pines, and the cultures were identical with the original

parent isolate (4). *T. terrestris* was not reisolated from mycorrhizae of Austrian, ponderosa, or lodgepole pines.

The degree of mycorrhizal development by *T. terrestris* varied among hosts and isolates, but variation was minimal within host–isolate combinations. Typical mycorrhizae formed by *T. terrestris*, regardless of isolate or host species, were macroscopically and microscopically indistinguishable. Most mycorrhizae were simple coralloid, but a few were either bifurcate or complex coralloid in structure. Fungal mantles were light brown to cream and from 4 to 21 μ thick (average 10 μ). Hartig-nets usually developed to the endodermis. Hyphal strands, with numerous clamp connections, connected adjacent mycorrhizae, and radiated from mycorrhizae into the rhizosphere as previously illustrated (6).

Pisolithus tinctorius isolate 49 formed varying amounts of ectomycorrhizae with 14 pine species (Table I). Atypical mycorrhizae were not formed by this fungal symbiont on any host. Mycorrhizae were primarily complex coralloid to nodular with many having more than 10 root points per mycorrhiza. Fungal mantles were gold brown and well developed, from 18 to 83 μ thick (average 36 μ). The Hartig-net developed to, or within one cortical cell layer of, the endodermis (Fig. 1). The mycorrhizae were morphologically indistinguishable, on all species, from those reported in aseptic pine culture (6). The fungal symbiont was reisolated from mycorrhizae of Japanese red, sand, Scots, Virginia, and shortleaf pines, and cultures were identical with the original parent isolate. *P. tinctorius* was not reisolated from mycorrhizae of loblolly or cluster pines.

Discussion

This report extends the verified host range of *T. terrestris* from 11 (3) to 21 pine species, Norway spruce, and Douglas fir, and extends the host range of *P. tinctorius* from 2 (1, 5) to 14 pine species. These conifers have a wide geographic distribution and represent many of the world's important coniferous tree species. The occurrence of *T. terrestris* in forest nurseries (10), and its ability to colonize fumigated (7) or other symbiont-free soils rapidly (13) by basidiospores (7), strongly suggest that it is an important component of the mycorrhizal complement of nursery and outplanted conifer seedlings throughout the world.

The formation of atypical (i.e. without fungal mantle) ectomycorrhizae by *T. terrestris* suggests that its symbiotic–parasitic potential varies with hosts, as reported by Melin with other fungal symbionts (9). Varying parasitic potential may account for the absence of mycorrhizal development on a host (such as Norway spruce or Himalayan pine) by one isolate of *T. terrestris* and the formation of atypical mycorrhizae on the same host by another isolate. Both isolates formed atypical mycorrhizae with Japanese red pine. Environmental factors, affecting both the host and symbiont, undoubtedly influence this type of mycorrhizal development. More research will determine if ectomycorrhizae lacking fungal mantles are characteristic of these particular symbiotic associations.

The lack of mycorrhizal development on certain conifer hosts by *T. terrestris* and *P. tinctorius* does not necessarily mean that these fungi are not potentially symbiotic with these hosts. This potentiality is especially exhibited by *P. tinctorius*, which has a high temperature requirement. Soil temperatures during this experiment averaged 21 °C, well below the optimum of 34 °C reported for maximum mycorrhizal synthesis by this symbiont on loblolly pine.[4] Maintenance of a higher soil temperature would have been more favorable for mycorrhizal development by *P. tinctorius* on these hosts.

The typical mycorrhizae formed by *T. terrestris* on the various conifer hosts were easily distinguished macroscopically and microscopically from those formed by *P. tinctorius*, but typical mycorrhizae formed by either isolate of *T. terrestris* were morphologically indistinguishable from each other, regardless of host. These observations confirm our earlier conclusion (3) that the symbiont exerts controlling influence on color and morphology of ectomycorrhizae. This information should simplify field evaluation of ectomycorrhizae formed by these symbionts, since characteristics of mycorrhizae formed by these fungus symbionts under essentially the

[4]Footnote 3.

same environmental conditions should be similar on different hosts.

This is the first report of pure culture synthesis of ectomycorrhizae on bristlecone, jack, lodgepole, Himalayan, and Austrian pines.

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